Establishment and application of endosperm culture systems to produce polyploid plants in Amaryllidaceae

Arisa Nakano
Graduate school of Environmental Science, Hokkaido University

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Chapter 1: Introduction and objectives of the research

There are many species of perennial bulbous plants, such as *Narcissus*, *Lycoris*, and *Crinum*, that have attractive flowers. Therefore, these plants are widely cultivated and used as ornamental plants. However, flowering requires the full development of the bulb, and it takes several years or more from seed to flowering. For this reason, they are generally propagated vegetatively.

Polyploid breeding is one of the breeding strategies. Polyploid plants with three or more sets of chromosomes show more vigorous growth and larger organs compared with diploid plants. Since their characteristics are useful horticulturally, the production of polyploid plants has been attempted in many plant species. In general, chromosome doubling by mitotic inhibitors such as colchicine and oryzalin is used to artificially produce polyploid plants with even number sets of chromosomes such as tetraploid and octoploid. In addition, interploid hybridization with a cross between diploid and tetraploid are required to produce polyploid plants with odd number sets of chromosomes such as triploid. However, there are two problems with interploid hybridization. First, it is necessary to produce tetraploid plants in advance and to regulate the flowering of diploid and tetraploid plants simultaneously. The second is the interploid hybridization barrier, which causes seed abortion due to abnormalities in endosperm development. Because of these issues, the production of polyploid plants with odd number sets of chromosomes requires a longer period and more labor than the production of polyploid plants with even number sets of chromosomes.

As a method of polyploid plant production, endosperm culture has been attracting attention. The endosperm of diploid plants in angiosperms is generally a triploid tissue formed by the fusion of a binucleate central cell and a haploid sperm cell. Therefore, it is possible to produce triploid plants from the seeds crossed from diploid plants by endosperm culture, which leads to a significant reduction in the breeding period. However,

almost 30 species of triploid plants have been produced from nearly 70 species of plants so far, and it is considered technically difficult to produce triploid plants by endosperm culture.

The objectives of this study were 1) to establish endosperm culture systems in Amaryllidaceae and 2) to expand the availabilities of endosperm culture. As described above, Amaryllidaceae members have long-term life cycles, which it makes very effective to shorten the period of polyploid plant production by endosperm culture. Previous studies on endosperm culture in monocots have been limited to Poaceae, and the establishment of endosperm culture systems in Amaryllidaceae was also expected to be a useful point for application to other monocotyledonous plants such as Liliaceae. In addition, I tried to produce not only triploid but also other polyploid plants by applying the established endosperm culture systems. I hope that the present study will improve the availability of endosperm culture, promote research, and lead to the investigation of factors that make endosperm culture technically difficult in the future.

Chapter 2: Establishment of endosperm culture systems in Amaryllidaceae

In Chapter 2, we first attempted the callus induction from endosperm in 27 plant species of 16 genera in the subfamily Amaryllidoiceae (the family Amaryllidaceae). The culture medium was used Murashige and Skoog (MS) medium supplemented with picloram as auxin and 6-benzylaminopuline (BAP) as cytokinin. As the result of endosperm culture, calli were induced in 10 species of six genera. To investigate the origin of the calli from endosperm tissue, the relative nuclear DNA content was measured by flow cytometer using diploid plants as an internal standard to confirm whether the ploidy of callus was the same as that of endosperm tissue. Flow cytometric analysis revealed the calli obtained from *Cyrtanthus mackenii*, *Haemanthus albiflos*, *H. pauculifolius*, and *Scadoxus multiflorus* were triploid and considered to be derived from the endosperm, indicating endosperm culture is available for Amaryllidaceae members.

Next, I evaluated the stability of endosperm culture in *H. albiflos*, which showed the highest callus induction rate, for the application of the endosperm culture system. The callus induction and shoot regeneration rates were examined in terms of seed, endosperm maturity, parts of endosperm, and medium composition. As a result, stable callus induction and shoot regeneration were observed, although some variation was observed depending on the origin of the seeds.

In this chapter, I established the endosperm culture systems available for four species of three genera in Amaryllidaceae: *C. mackenii*, *H. albiflos*, *H. pauculifolius*, and *S. multiflorus*. Furthermore, the stability of callus induction and shoot regeneration was indicated by the investigation of the effect of culture condition on endosperm culture in *H. albiflos*. Therefore, *H. albiflos* is considered a useful material to investigate the application of endosperm culture to produce polyploid plants.

Chapter 3: Endosperm-derived triploid plant regeneration and hexaploid plant production from endosperm-derived callus treated with colchicine in diploid *Haemanthus albiflos*

In Chapter 3, to expand the availability of endosperm culture, I attempted to produce hexaploid plants by the established endosperm culture system of *H. albiflos*. The production of hexaploid plants aims to restore the fertility of triploid plants which show sterility due to unbalanced chromosome pairing. I treated triploid endosperm-derived calli with colchicine, a mitotic inhibitor, and cultured them on the shoot regeneration medium. Then, the shoot regeneration pathway was also investigated by histological observation of the calli regenerating shoots. The shoots regenerated from the treated calli grew into plantlets. Ploidy analyses of flow cytometry and chromosome counts revealed the presence of hexaploid plants among the regenerated plantlets, indicating that hexaploid plants could be produced from the endosperm crossed from diploid plants by a combination of endosperm culture and colchicine treatment. Histological observations revealed that the endosperm-derived calli retained two regeneration pathways of somatic embryogenesis and adventitious shoot organogenesis.

In this chapter, I showed a new example of plant regeneration from endosperm tissue through somatic embryogenesis and adventitious shoot organogenesis in *H. albiflos*. Furthermore, hexaploid plant production was demonstrated by the combination of endosperm culture and colchicine treatment. Thus, this study provides a method for the simultaneous production of triploid and hexaploid plants from the endosperm crossed from diploid plants, which will be expected to be utilized for polyploid breeding.

Chapter 4: Production of tetraploid and octoploid plants from immature embryo-derived callus treated with colchicine in *Haemanthus albiflos*

In Chapter 4, I attempted to produce tetraploid and octoploid plants from diploid immature embryos obtained in the process of the endosperm culture of Chapter 3. The immature embryos were cultured on MS medium supplemented with picloram and BAP

as callus induction from the endosperm. After colchicine treatment to the immature embryo-derived calli, shoot regeneration was induced on 1/2MS medium without plant growth regulators. The regeneration pathway from the calli was investigated by histological observation of regenerated shoots. Ploidy analyses of regenerated plantlets revealed that tetraploid and octoploid plants regenerated from the treated calli. Previous studies have been reported that mixoploids, which have cells of different ploidy levels and reduce the efficiency of polyploid plant production. In this study, there was only one mixoploid, and the efficiency was high. It was thought to be related to the regeneration pathway by somatic embryogenesis.

In this chapter, I showed the immature embryo obtained from endosperm culture in Chapter 3 available to produce tetraploid and octoploid plant production. Thus, various polyploid plants including triploid (3x), tetraploid (4x), hexaploid (6x), and octoploid (8x) enable to be produced by the seeds crossed diploid (2x) plants in *H. albiflos.*

Chapter 5: General discussion

The present study demonstrated that diploid, triploid, tetraploid, hexaploid, and octoploid plants could be produced from seeds crossed from diploid plants by the establishment of endosperm culture system and the application. This is expected to contribute to polyploid breeding because it can overcome the long-term production and the interploid hybridization barrier, which are problems in polyploid breeding. In addition, the established endosperm culture system of *H. albiflos* in this study is capable of stable callus induction and plant regeneration. It is expected to contribute to the elucidation of plant regeneration mechanisms in endosperm tissue through molecular genetic research.